# Splice Variants of Aryl Hydrocarbon Receptor in the Dioxin Resistant Rat: Tissue Expression and Transactivational Activity

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## Introduction

The *AHR* locus encodes a ligand-activated transcription factor, the Aryl Hydrocarbon Receptor (AHR), which plays roles in: 1) adaptive metabolism of xenobiotics; 2) developmental and physiological signaling; and 3) toxic response to xenobiotics. Ligand binding converts the AHR into a heterodimeric complex that regulates expression of specific genes via binding directly to AH responsive elements (AHREs). The AHR is ubiquitously expressed in vertebrate cells and tissues, suggesting that it has a conserved physiological signaling role. The AHR also mediates virtually all toxic effects of dioxin-like environmental pollutants; *Ahr*-null mice are highly resistant to TCDD toxicity(1). Structural changes in the AHR that block nuclear translocation(2) or reduce affinity for ligand binding(3) reduce susceptibility to dioxins. Depletion of the AHR's binding partner (ARNT) also results in phenotypic alterations mimicking that of the *Ahr*-null mouse(4). This combined evidence indicates that both partners of the heterodimerization transcription factor complex are essential components of the toxic response to dioxins.

The Han/Wistar (*Kuopio*) (H/W) rat [LD50>9600 µg/kg] is >1000-fold resistant to acute TCDD lethality compared with the Long-Evans (*Turku AB)* (L-E) rat strain [≈10 µg/kg](5) although several non-lethal effects, pharmacokinetics and P450 induction are very similar(6,7). The AHR in H/W rats carries a large deletion in the transactivation domain (TAD) that appears to protect from lethal effects of TCDD. Specifically, a point mutation in the intron/exon 10 boundary leads to use of 3 cryptic splice sites potentially creating 3 alternative transcripts and 2 possible protein products(8) (Figure 1). There are no major differences in the heterodimerization partner ARNT protein between the resistant H/W and sensitive L-E rats(9). Multiple crosses between H/W and L-E rat strains (combined with TCDD challenge) generated one additional sensitive line (Line-C, "LnC") and two resistant groups (F1 and Line-A, "LnA")(6,10). Our **objective** is to quantitatively determine which of the alternative splice variant products are expressed in the resistant rat in contrast to the sensitive rats. Identification of the specific expressed genetic variant (s) in resistant rats will help to define the key regions of the AHR that potentially are important for differential gene regulation and inherent resistance to dioxin toxicity *in vivo.* Further, since the function of the AHR's TAD is to transactivate gene expression we measured, *in vitro*, the ability of each of the H/W rat's alternative AHR splice variants to transactivate gene expression in comparison to the AHR of L-E.

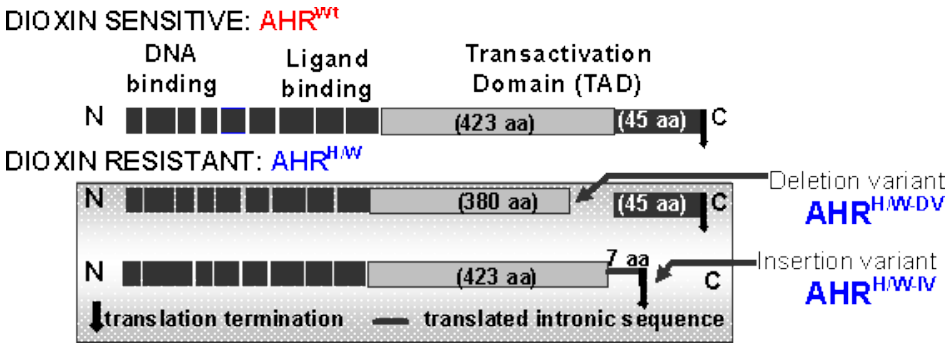


***Figure 1:*** Alternative splicing: H/W variant and wild-type AHR protein structure[9].

**Materials and Methods**

***Animal treatment & isolation of total RNA***-TCDD susceptible rats: L-E, LnC and, Sprague Dawley (SD) rats in addition to TCDD-resistant rats: H/W, LnA, and $F_1$ [L-E x H/W] (male; 10-12 weeks old), were from the breeding colony of the National Public Health Institute, Division of Environmental Health, Kuopio, Finland. All rats were given 100 µg/kg TCDD or corn oil vehicle by gavage then euthanized by decapitation after 3 hours, 19 hours or 4 days (4 animals per group). Total RNA was extracted from liver using Qiagen's RNeasy kits according to the manufacturer's instructions. Total RNA from kidney, lung, testis and thymus was isolated using TRIzol reagent (Invitrogen) and subsequently treated with DNase (MBI Fermentas).

***Expression and reporter constructs***-We previouslygenerated expression constructs forthe wild-type ($AHR^{Wt}$) and the 2 variant receptor forms ($AHR^{H/W-IV}$ or $AHR^{H/W-DV}$). The TAD of each AHR variant was cloned from its respective full-length AHR expression construct and inserted into pFA-CMV (Stratagene) in-frame with the GAL4-DNA binding domain (GAL4-DBD-*BamHI*-**AHRTAD**-*HindIII*). pFR-LUC (Stratagene) was used as a reporter of transactivation activity of the GAL4-**AHRTAD** chimera. Briefly, interaction of the GAL4-DBD with a UAS consensus element of the reporter construct facilitates association of the GAL4-**AHRTAD** with the promoter to drive fireflyluciferase expression. The resulting induction is directly proportional to intrinsic transactivation activity of the **AHRTAD**. pRL-TK (Promega) encoding renilla luciferase was used as a control for transfection efficiency.

***Cell Culture / in vitro assays***-Rat hepatoma cells (5L) were transfected with 2 µl Lipofectamine and 2 µl Plus reagent (Invitrogen), mixed with0.15 µg of one of the three GAL4-**AHRTAD** chimeras, 0.115 µg pFR-LUC and 0.035 µg pRL-TK. Cells were harvested 24 hours later and assayed for both firefly and renilla luciferase activity using the Dual Luciferase Assay (Promega).

***Real-time quantitative RT-PCR***-Three allele-specific primer/probe sets were designed to uniquely amplify each of the 2 variant forms of the H/W receptor in addition to the wild-type receptor. The specificity of each primer/probe was confirmed by: 1) sequencing of PCR products amplified from liver, 2) positive amplification from a construct containing the specific cDNA of one variant and 3) negative amplification from constructs containing the other AHR cDNAs. Total RNA (2 µg) was reverse transcribed and amplified using real-time PCR (Stratagene MX4000). Absolute AHR transcript levels for each individual rat were determined using a standard curve of a 10-fold serial dilution of each construct ($AHR^{Wt}$ or $AHR^{H/W-IV}$ or $AHR^{H/W-DV}$) in triplicate.

**Results and Discussion**

***1) Which of the alternative AHR splice variant transcripts are expressed in the resistant rat?*** Untreated dioxin-resistant rats (H/W & LnA) express greater amounts of the $AHR^{H/W-IV}$transcript than the $AHR^{H/W-DV}$ and express no detectable levels of $AHR^{Wt}$ transcript in all tissues examined. Dioxin-sensitive rats (L-E, SD & LnC) express the $AHR^{Wt}$transcriptin all tissue types examined; thymus and liver do express detectable $AHR^{H/W-IV}$ mRNA but at very low levels. Therefore, it appears that the splice site used by the resistant rat also can function to a limited extent in sensitive animals but is not the predominant splice site. In liver of F1 offspring of an L-E x H/W cross each of the 3 transcripts is expressed; the $AHR^{H/W-IV}$ transcript is equal to $AHR^{Wt}$ but very low $AHR^{H/W-DV}$ transcript levels were

detected. It appears that having at least one copy of the $AHR^{H/W-IV}$ allele is sufficient to make the F1 rat resistant to dioxin toxicity. A possible explanation is that in the F1 rat the presence of the variant $AHR^{H/W-IV}$ protein out-competes $AHR^{Wt}$ for co-activators/enhancers. In kidney, testis and lung L-E rats express ~2-fold greater total $AHR^{Wt}$ mRNA levels than the other 5 strains/lines of rats but no significant difference was observed between strains/lines in thymus, or liver. Previous studies report a ~ 2-fold higher lung and hepatic AHR protein concentration in L-E rats than H/W rats [6,11]. There is no apparent correlation between overall receptor abundance and sensitivity to dioxin among the 6 rat strains/lines investigated. The rank order of receptor mRNA abundance in the various rat tissues is lung>thymus>kidney> liver>testis. Elevated levels of AHR in lung are in agreement with AHR's role as an environmental sensor as the lung is in direct contact with the environment.

***2) Does expression of the alternative AHR splice variant transcripts change with time or dioxin treatment?*** TCDD treatment (100 µg/kg, 19 hr) had no significant effect on AHR mRNA expression in either L-E or H/W rat livers (p=>0.2, n=3). The lack of a clear relationship between patterns of AHR regulation by TCDD and toxic endpoints seen thus far indicate that inherent strain susceptibility is likely not attributable to differential regulation of the AHR by TCDD, in agreement with Franc et al.[11]. Further, TCDD exposure did not alter the expression pattern of the alternative $AHR^{H/W}$ splice variant transcripts in the liver ($AHR^{H/W-IV}$ >>> $AHR^{H/W-DV}$). The extraordinary resistance of the H/W rat to lethal effects of TCDD can not be attributed to alteration of the level of the alternative splice variants. We are currently quantifying the effect of TCDD exposure on regulation of the $AHR^{Wt}$ and $AHR^{H/W}$ variants in other rat tissues for all 6 strains/lines at an early (3 hr) and a late (4 day) time points. Previous reports *in vivo* on the effect of TCDD exposure on AHR levels have been contradictory. Therefore, obtaining an accurate representation of AHR mRNA levels in individual tissues will clarify the effect of TCDD on AHR mRNA expression and could point to a particular tissue as being crucial to understanding dioxin toxicity.

***3) What are the functional consequences of the variant forms of the AHR?*** The intrinsic activity of the $AHRTAD^{H/W-DV}$ was significantly higher than either the $AHRTAD^{H/W-IV}$ or the $AHRTAD^{Wt}$. The intrinsic activity of the$AHRTAD^{H/W-IV}$ and $AHRTAD^{Wt}$did not significantly differ. It is unclear what impact this difference in intrinsic transactivation will have on endogenous genes regulated by the AHR. However, it provides preliminary evidence that the region perturbed in the Han/Wistar AHR variant(s) may play a functional role in the differential expression of AHR-regulated genes observed in our *in vivo* studies.

**Significance and Future Studies:** The AHR is the first essential component in the dioxin toxicity cascade. Therefore understanding the qualitative and quantitative influences of alternative splice variants at the mRNA level will have an important impact on our understanding of differential sensitivity to dioxins. Our measurements of transcript levels *in vivo* and measurement of transactivation activity *in vitro* describe only part of the story. Future studies will examine the effect of TCDD exposure on $AHR^{H/W}$ variant protein expression in resistant rats. Further, despite the sizeable deletion of the AHR's TAD, *CYP1A1* induction remains normal in H/W rats[7].We **hypothesize** that this deletion in the $AHR^{H/W}$ TAD disrupts transactivation of a specific set of genes potentially relevant to dioxin toxicity. We are testing this hypothesis using candidate genes previously identified as commonly regulated and genes that are differentially regulated by TCDD between dioxin-sensitive versus dioxin-resistant rats[12].

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